AMENDMENTS TO THE SPECIFICATION

Please delete the paragraph on page 6, line 15 to page 7, line 30 and replace it with the following paragraph:

One of the targets selected with Efficacy-First, tumor rejection antigen-1 (TRA-1), was found to have increased expression in tumors induced to accelerated growth. TRA-1, also known as glucose-regulated protein 94 (grp94), gp96, endoplasmiin precursor and other names, was first described as a molecular chaperone [Hartl FU. (1996) Molecular chaperones in cellular protein folding. *Nature* 381(6583):571-9] with important roles in endoplasmic reticulum related to nuclear signaling, protein folding, sorting and secretion [Nicchitta, C.V. (1998): Biochemical, cell biological and immunological issues surrounding the endoplasmic reticulum chaperone GRP94/gp96. *Current Opinion in Immunology,* 10:103-109.]. In addition, it exerts a specific protection against Ca2+ depletion stress and is involved in antigen presentation [Tamura, Y. P. Peng, K. Liu, M. Daou, P.K. Srivastava, 1997: Immunotherapy of tumors with autologous tumor-derived heat shock protein preparation. *Science,* 278:117-120]. Furthermore, it also has an important role in tumorigencity [Udono H, Levey DL, Srivastava PK. (1994) Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8=T cells in vivo. *Pro Natl Acad Sci* USA 91: 3077-3081.]. Menoret et al. [Menoret A, Meflah K, Le Pendu J. (1994) Expression of the 100 kDa glucose-regulated protein (GRP100/endoplasmin) is associated with tumorigenicity in a model of rat colon adenocarcinoma. *Int J Cancer* 56: 400-405] reported that there was an overexpression of TRA-1 in a model of rat colon adenocarcinoma. Gazit et al. [Gadi Gazit, Jun lu, Amy S.Lee. (1999) De-regulation of GRP stress protein expression in human breast cancer cell lines. *Breast Cancer Research and Treatmen*t 54: 135-146.] found out there was a 3-5 fold increase in the level of TRA-1 protein was observed in five human breast cancer lines as compared to the normal human mammary lines. Cai et al. [Cai JW. Henderson BW, Shen JW, et al (1993) Induction of glucose-regulated proteins during growth of murine tumor. *J Cell Physiol* 154; 229-237] found through studies during

growth of tumors that the level of the TRA-1 is increased, correlating with the size of the tumor. Elevated level of TRA-1 has been implicated to protect neoplastic cells and tumors against cytotoxic T-lymphocyte mediated cytotoxicity and protected tissues culture cells against adverse physiological conditions [Sugawara S, Takeda K, Lee A, et al. (1993) Suppression of stress protein GRP78 induction in tumor B/C10ME eliminates resistance to cell mediated cytotoxicity. *Cancer Research.* 53: 6001-6005]. Public domain databases reveal that TRA-1 is over-expressed in many human cancer tissues including prostate, mammary, brain, stomach, and soft tissue tumors. Overexpression, antisense and ribozyme approaches in tissue culture system directly showed that TRA-1 could protect cells against cell death [Little E, Ramakrishnan M, Roy B, et al. (1994) The glucose-regulated proteins (GRP78 and GRP94): Functions, gene regulation, and applications. *Crit Rev Eukaryot Gene Expr* 4: 1-18, Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. (2001). Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem *Biophys Res Commun.* 286(3):433-42., Ramachandra K. Reddy, et al. (1999). The endoplasmic reticulum chaperone glycoprotein GRP94 with Ca2+-binding and antiapoptotic properties is a novel proteolytic target of calpain during etoposide-induced apoptosis. *J. Biol. Chem* 274: 28476-28483]. These anti-apoptosis effects of TRA-1 are associated with induction in neoplastic cells and may lead to cancer progression and chemotherapy resistance. Although normally confined to the ER, TRA-1 has been shown to escape to KDEL **(SEQ ID NO: 103)**-mediated retention system in several cell types. For instance, a significant fraction of TRA-1 is secreted to the extracellular space by hepatocytes and exocrine pancreatic cells, via the normal secretory pathway. In several tumor cell lines TRA-1 is detectable as an outer surface protein [Altmeyer A, Maki RG, Feldweg AM, Heike M, Protopopov VP, Masur SK, Srivastava PK (1996). Tumor-specific cell surface expression of the-KDEL **(SEQ ID NO: 103)** containing, endoplasmic reticular heat shock protein gp96. *Int. J. Cancer* 22;69(4):340-9.].

Please delete the paragraph on page 14, lines 13-14 and replace it with

the following paragraph:

Figure 10 shows that expression of ICT1024 in breast tumor tissue has significantly positive correlation with **other breast cancer genes and** other cancer genes, based on the SAGE/microarray analysis.

Please delete the paragraph on page 14, lines 17-19 and replace it with the following paragraph:

Figure 12 shows that ICT1024 protein has significant structural homology to other rhomboid proteins from various organisms, such as yeast, bacteria and plant. (SEQ ID NOs: 27 – 35**, respectively in order of appearance**)

Please delete the paragraphs on page 16, line 17 to page 18, line 6 and replace it with the following paragraphs:

~~Figure 33 shows the sequence of ICT1025 cDNA, Genebank Accession No. NM_003299, Tumor Rejection Antigen 1 or gp96. (SEQ ID NO:70)~~

~~Figure 34 shows the sequence of ICT1025 peptide, NP_003290 (SEQ ID NO:71), named as Tumor Rejection Antigen (gp96) 1, Glucose Regulated Protein, grp94 and Endothelial cell glycoprotein.~~

~~Figure 35 shows that the ICT1025 siRNA Design: two 21 nt sequences from ICT1025 were selected as the targets for RNAi-mediated knockdown of ICT1025 gene expression. (SEQ ID NO:72 and 73)~~

~~Figure 36 shows that ICT1025 specific siRNA duplexes are able to knockdown TRA-1 expression in MDA-MB-435 cells, at both message RNA level detected with RT-PCR and protein level detected with Western blot analysis. The knockdown of ICT1025 gene expression with siRNA has demonstrated dose dependent effects.~~

~~Figure 37 shows that ICT1025 specific siRNA duplexes induce apoptosis activity of MDA-MB-435 cells observed at 48 hours after the transfection.~~

~~Figure 38 shows that ICT1025 specific siRNA duplexes decrease cell proliferation of HT-29 cells observed 48 hours after the transfection.~~

~~Figure 39 shows that ICT1025 specific siRNA duplexes induce apoptosis activity of HT-29 cells observed at 48 hours after the transfection.~~

~~Figure 40 shows that ICT1025 specific siRNA duplexes inhibit growth of MDA-MB-435 cell formed xenograft tumor on nude mice using repeated delivery of the siRNA duplexes. The inhibition of tumor growth caused by ICT1025 knockdown is much stronger than that of hVEGF knockdown.~~

~~Figure 41 shows that when the commercial monoclonal antibody specific to ICT1025 was applied on the MDA-MB-435 cells the apoptosis activity of the cells increased dramatically in a dose dependent manner.~~

~~Figure 42 shows that ICT1025 is located in the membrane fraction of the cell lysates from both MDA-MB-435 cells and MCF-7/VEGF165 cells, detected by the monoclonal antibody.~~

~~Figure 43 shows that not only the ICT1025 is located in the membrane fraction, but also presented the extracellular domains on the cell surface, detected by the monoclonal antibody binding of the biotinylated surface proteins.~~

~~Figure 44 shows that upregulated expression of ICT1025 in multiple cancer tissues illustrated with a virtual Northern analysis using SEGE database published by NCI.~~

~~Figure 45 shows the domain architecture of ICT1025 with a Head Shock Protein 90 domain and a human ATPase_c domain.~~

~~Figure 46 shows that peptide sequence homology between human TRA-1 and mouse TRA-1 (SEQ ID NO: 71 and 74, respectively). The two proteins are highly similar.~~

~~Figure 47 shows that the peptide sequence homology between TRA-1 and head shock protein 90 (SEQ ID NO:71 and 75, respectively).~~

~~Figure 48 shows the predictions of the transmembrane structure of ICT1025.~~

~~Figure 49 shows the prokaryotic expression vector PGEX53X1025 carrying full-length sequence of ICT1025.~~

~~Figure 50 shows the purified ICT1025 protein expressed from the prokaryotic system.~~

~~Figure 51 shows the eukaryotic expression vector pCI-ICT1025 carrying the full-length cDNA of ICT1025.~~

Figure [[52]]**33** shows HLa peptide motif search results **(SEQ ID NOS 83-102, respectively in order of appearance)**.

Figure [[53]]**34** shows suggested models for transmembrane biology of ICT 1025.

Figure [[54]]**35** shows predicted transmembrane segments of ICT 1025.

Figure [[55]]**36** shows screening of ICT 1025 mAB for surface binding activities in breast tumor cells.

Figure [[56]]**37** shows screening of ICT1025 mAB for surface binding activities in colon tumor cells.

Figure [[57]]**38** shows inhibition of tumorigenesis and tumor growth by treating tumor cells with antibody or siRNA prior to inoculation.

Please delete the paragraph on page 30, lines 19-33 and replace it with the following paragraph:

Tumor tissues was obtained and used to isolate total RNA for microarray analysis (Affymetrix, U133). One of the highly un- or down-regulated genes (about 1% of the total probes on the U133 chip), ICT1024, demonstrated significant up-regulated expression with signal from 585 (control group expression level), to 1208 (treated group expression level). This gene was therefore selected for the next level of target validation with a method called Disease-Control™ validation, using an siRNA based in vivo knockdown in the same xenograft tumor model. Two siRNA duplexes, 21 base pair each (Fig. 7) (SEQ ID NO: 25 and 26), were designed targeting this ICT1024 gene, specific to the sequence of AK026010, NM_022450 and M99624, in the coding region (aagctggacattccctctgcg **(SEQ ID NO: 26)**, aagagcccagcttcctgcagc **(SEQ ID NO: 25)**). Then the two siRNA duplexes were delivered intratumorally three times. The siRNA-

mediated knockdown of ICT1024 gene expression resulted in tumor growth inhibition (Fig. 8). The further analysis in a cell culture based study demonstrated that knocking down ICT1024 gene expression in the tumor cell MDA-MB-435, induced a remarkable increase of the apoptosis activity (Fig. 9). Based on these results, ICT1024 was selected for further evaluation as a therapeutic target.

Please delete the paragraph on page 32, lines 11-27 and replace it with the following paragraph:

Tumor tissues was obtained and used to isolate total RNA for microarray analysis (Affymetrix, U133). One of the highly un- or down-regulated genes (about 1% of the total probes on the U133 chip), ICT1025, demonstrated significant up-regulated expression with signal from 279 (control group expression level), to 412 (treated group expression level). This gene (Accession No. AK025852, NM_003299 and BC009195~~, Figure 33, mRNA sequence (SEQ IDNO:70); Figure 34 protein sequence (SEQ ID NO:71)~~) was therefore selected for the next level of target validation with a method called Disease-Control™ validation, using an siRNA based in vivo knockdown in the same xenograft tumor model. Two siRNA duplexes, 21 base pair each~~ (Fig. 35) (SEQ ID NO. 72 and 73)~~, were designed targeting this ICT1025 gene, specific to the sequence in the coding regions of aactgttgaggagcccatgga (started at nt 966) **(SEQ ID NO: 23)** and aatctgatgatgaagctgcag (started at nt 1008) **(SEQ ID NO: 24)**. Then the two siRNA duplexes were delivered intratumorally three times. The siRNA-mediated knockdown of ICT1025 gene expression resulted in tumor growth inhibition~~ (Fig. 36)~~. The further analysis in a cell culture based study demonstrated that knocking down ICT1025 gene expression in the tumor cells MDA-MB-435, HT29 induced remarkable increases of the apoptosis activity~~ (Fig. 37)~~ and decrease of cell proliferation. Based on these results, ICT1025 was selected for further evaluation as a therapeutic target.

Please delete the paragraph on page 35, lines 7-20 and replace it with the

following paragraph:

We also analyzed the hydrophobicity of ICT1024 protein and its potential trans-membrane location. Multiple prediction program have been applied, including SOSUI model (Table III **(SEQ ID NOS 106-111, respectively in order of appearance)**), TMHMM Model and TMpred Models (Fig. 15). From those analyses, it seems that ICT1024 is a integral membrane protein with multiple transmembrane domains and intracellular domains and extracellular domains. Whatever methods used for the predication of the protein location and topology, this protein has been demonstrated with a long N-terminal domain outside the membrane. This N-terminal domain would either be outside of cell or inside the cytoplasm. There are other regions of this protein also exposed to the outside of the cell or cytoplasm. The membrane protein, ICT1024, has a proteinase activity for activation of EGF-EGF receptor pathway and, based on the discoveries described herein, is a very attractive target for therapeutic development of various modalities of drugs, including monoclonal antibody, siRNA inhibitor, peptide antagonist and small molecular inhibitors, etc. A suitable monoclonal antibody will bind to either the extracellular or intracellular domain of the protein and block function of the protein.

Please delete the paragraph on page 37, line 31 to page 38, line 26 and replace it with the following paragraph:

In the Drosophila cell, the polytopic membrane protein Rhomboid-1 promotes the cleavage of the membrane-anchored TGFalpha-like growth factor Spitz, allowing it to activate the Drosophila EGF receptor. Until now, the mechanism of this key signaling regulator has remained obscure, but this analysis suggests that Rhomboid-1 is a novel intramembrane serine protease that directly cleaves Spitz. In accordance with the putative Rhomboid active site being in the membrane bilayer, Spitz is cleaved within its transmembrane domain, and thus is the first example of a growth factor activated by regulated intramembrane proteolysis. Rhomboid-1 is conserved throughout evolution from archaea to humans, and these results show that a human Rhomboid

promotes Spitz cleavage by a similar mechanism. This growth factor activation mechanism may therefore be widespread (6). Although Rhomboid-1 does not contain any obvious sequence homology domains, it has the characteristics of a serine protease (7). Four of its six essential residues parallel the residues required for a serine protease catalytic triad charge-relay system and an oxyanion stabilization site (consisting of a glycine two residues away from the active serine, and the serine itself; G215 and S217). These are the two active site determinants of serine proteases, and these four essential residues account for all of the amino acids known to participate directly in the serine protease catalytic mechanism (5) These residues are absolutely conserved in all Rhomboids, and their mutation to even very similar residues (i.e., G215A, S217T, and S217C) abolishes Rhomboid-1 activity. These are hallmarks of active site residues. (3) The location of the essential residues is highly suggestive of a serine protease active site; both G215 and S217 occur in the conserved GASGG motif **(SEQ ID NO: 104)**, which is remarkably similar to the conserved GDSGG motif **(SEQ ID NO: 105)** surrounding the active serine of 200 different serine proteases. Furthermore, the essential residues N169 and H281 occur at the same height in their transmembrane domains (TMDs) as the GASGG motif **(SEQ ID NO: 104)**, consistent with the proposal that they associate with S217 to generate a catalytic triad. Finally, Spitz processing is directly inhibited by the specific serine protease inhibitors DCI and TPCK, and Rhomboid-1 itself becomes limiting in their presence, suggesting that Rhomboid-1 is their direct target and thus the serine protease responsible for Spitz cleavage. The suggested model is presented in Figure 16.

Please delete the paragraphs on page 40, line 9 to page 41, line 2 and replace it with the following paragraphs:

The protein ICT1025 apparently plays a key role in tumor metastasis and tumor growth, through its multiple roles as inhibitor of apoptosis, activator of proliferation and up-regulation of multiple drug resistant genes. We have evidence to demonstrate that this gene is up-regulated in fast growing tumor from a xenograft tumor model study

treated with bFGF expression vector. This gene is up-regulated in mRNA level in tumor tissues, from breast cancer, prostate cancer, brain cancer and other types of cancers, based on the SAGE virtual and digital northern analyses. This gene has also been shown to be up-regulated using Gene Logic's GeneExpresse analysis. When the gene expression was knockdown with ICT1025 specific siRNA duplexes in the growing xenograft tumors, the tumor growth was significantly inhibited ~~(Fig. 38)~~.

Apoptosis (programmed cell death) is a form of cellular suicide that typically includes plasma membrane blebbing, cellular volume contraction, and nuclear condensation, and culminates in the activation of endogenous endonucleases that degrade cellular DNA. The well-defined loss of specific cells is crucial during embryonic development and organogenesis. In addition to its physiological roles, apoptosis also occurs in many types of cancer cells when they are exposed to various chemotherapeutic drugs, including antimetabolites, deoxynucleotide synthesis inhibitors, DNA topoisomerase inhibitors, anti-microtubule agents, alkylating agents, and endoplasmic reticulum (ER) stressors. Interestingly enough, when we knockdown ICT1025 expression in MDA-MB-435 cells and HT-29 cells, transfected with the specific siRNA duplexes, the apoptosis activity was dramatically increased ~~(Figure 37 and 38)~~, as tested with a TUNEL assay, in which terminal deoxynucleotidyl transferase (TdT) catalyzes the incorporation of bromo-deoxyuridine (BrdU) residues into the fragmenting nuclear DNA at the 3'-hydroxyl ends by nicked end labeling.
The specific ICT1025 gene silencing by siRNA duplexes has been verified by RT-PCR ~~(Fig. 36)~~. This finding suggested that ICT1025 plays a crucial role in regulation of tumor cell apoptosis. Other evidence tends to show that the ICT1025 is sufficient to activate the major signaling pathways leading to cell proliferation and survival.

Please delete the paragraph on page 61, lines 16-19 and replace it with the following paragraph:

Targeted region (base position numbers 462-482, (SEQ ID NO:19) 5'-aatgacaagccacatcgatgt-3', and the corresponding sense siRNA (SEQ ID

NO:20) 5'-aa[[t]]ugacaagccaca[[t]]ucga[[t]]ug[[t]]u-3'; and continuing in this progression to the end of ICTB1003 coding sequence, as set forth herein.

Please delete the paragraph on page 87, line 30 to page 88, line 2 and replace it with the following paragraph:

Two cell lines, a human breast tumor cell line MDA-MB-435 and a human colon tumor cell line HT29, both of them over-expressing ICT1025 protein, were used in the mAb screening studies. **Culture supernatants from the 40 ICT1025 mAb secreting hybridoma clones were screened for cell surface binding activity in MDA-MB-435 and HT29 cells using a live cell surface staining ELISA assay (Figures 36 and 37). Mouse IgG at various concentrations were used as non-specific controls. Also, the supernatants from three GST mAb secreting hybridoma clones (2H2, 1H2, and 3G3) were used as negative controls (Figure 37).** The data from mAb screening using hybridoma culture supernatant in MDA-MB-435 cells (Figure [[55]]**36**) and HT29 cells (Figure [[56]]**37**) demonstrated that there are great variations in surface binding activies among the 40 mAb, with the data from one cell line confirmed the data from the other cell line. **The clones with the highest cell surface binding activities (Absorbance value) were selected for mAb purification.**

Please delete the paragraph on page 88, lines 8-13 and replace it with the following paragraph:

The effect of 1025 inhibition on tumorigenesis and tumor growth was determined by treating the human breast tumor cell line MDA-MB-435 with either agent and inoculating treated cells into nude mice. **For antibody treatment, 5 million MDA-MB-435 cells were pre-incubated with 100 ug of 1025 mAb or rat IgG in a total volume of 1 ml culture medium at 37°C for 4 hours. After washing with PBS, the cells were inoculated into the fat pads of nude mice at 0.4 million cells per site. For siRNA treatment, 5 million MDA-MB-435 cells were transfected with 10 ug of 1025 siRNA or GFP siRNA using electroporation. Then cells were incubated in a**

total volume of 1 ml culture medium at 37°C for 4 hours. After washing with PBS, the cells were inoculated into the fat pads of nude mice at 0.4 million cells per site. For the control group, 5 million MDA-MB-435 cells were incubated at 37°C for 4 hours. After washing with PBS, the cells were inoculated into the fat pads of nude mice at 0.4 million cells per site. The tumors formed from treated cells showed s[[t]]ubstantial inhibition in growth rate compared with cells treated by negative controls (Figure [[57]]**38**) and confirmed the 1025 inhibition effect on cells in culture also applies to tumorigenesis and tumor growth.